EXHIBIT F

Mechanochemical-hydrothermal preparation of crystalline hydroxyapatite powders at room temperature

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Crystalline hydroxyapatite (HAp) powders were prepared at room temperature from heterogeneous reaction between $Ca(OH)_2$ powders and $(NH_4)_2HPO_4$ solutions via the mechanochemical-hydrothermal route. X-ray diffraction, infrared spectroscopy, thermogravimetric characterization, and chemical analysis were performed, and it was determined that the room temperature products were phase-pure, thermally stable HAp with a nearly stoichiometric composition. Dynamic light scattering revealed that the dispersed particle size distribution of the room temperature HAp powders was in the range of 0.15–3.0 μm with a specific surface area of ≈90 m^2/g. Both specific surface area measurements and scanning electron microscopy confirmed that the HAp powders consisted of agglomerates containing hundreds of ≈20 nm HAp crystals.

Hydroxyapatite (HAp) with the chemical formula $Ca_{10}(PO_4)_6(OH)_2$ has been extensively used in medicine for implant fabrication and is one of the most biocompatible materials owing to its similarity with mineral constituents found in hard tissue (i.e., teeth and bones).[1–3] Multiple techniques have been used for preparation of HAp powders with wet methods[1,4–9] and solid state reactions[1,10] as the most popular. Depending upon the technique, materials with different morphology, stoichiometry, and level of crystallinity can be obtained. Recently, several papers regarding mechanochemical and mechanochemical-hydrothermal synthesis of HAp powders appeared in the literature.[11–18] Mechanochemical powder synthesis is a solid-state synthesis method that takes advantage of the perturbation of surface-bonded species by pressure to enhance thermodynamic and kinetic reactions between solids.[19] Pressure can be applied at room temperature by milling equipment ranging from low-energy ball mills to high-energy stirred mills (usually attrition, planetary, or vibratory). The main advantages of the mechanochemical synthesis of ceramic powders are simplicity and low cost.

Since the mechanochemical synthesis involves only solid-state reactions, it should be clearly distinguished from the *mechanochemical-hydrothermal* synthesis (sometimes called "wet" mechanochemical), which takes advantage of the presence of an aqueous solution in the system. An aqueous solution can actively participate in the mechanochemical reaction by acceleration of dissolution, diffusion, adsorption, reaction rate, and crystallization (nucleation and growth).[20] The mechanochemical activation of slurries can generate local zones of high temperatures (up to 450–700 °C) and high pressures due to friction effects and adiabatic heating of gas bubbles (if present in the slurry), while the overall temperature is close to the room temperature.[21] The mechanochemical-hydrothermal technique is thus located at the intersection of hydrothermal[20] and mechanochemical[19] processing. Correspondingly, if nonaqueous solutions were used we would define the process as *mechanochemical-solvothermal*. The mechanochemical-hydrothermal route produces comparable amounts of HAp powder as the hydrothermal processing, but it requires lower temperature, i.e., room temperature, as compared to 90–200 °C for the hydrothermal processing. Thus, for the mechanochemical-hydrothermal processing, there is no need for a pressure vessel and external heating.

The previous mechanochemical and mechanochemical-hydrothermal syntheses of HAp powders were usually accomplished at room temperature under either dry conditions or in water using either $CaHPO_4 \cdot 2H_2O$ or

$Ca(H_2PO_4)_2 \cdot H_2O$ as sources of calcium and phosphorus, $Ca(OH)_2$ and/or $CaCO_3$ as sources of calcium. The as-prepared powders were often nano-sized with considerable agglomeration.[15,16] The presence of $CaCO_3$ in the starting slurries resulted in the formation of strongly carbonated apatite powders.[14,16,17] A common feature of the HAp powders prepared in all the previous work was their very low crystallinity. Another disadvantage of the previously synthesized powders was their high nonstoichiometry (Ca/P molar ration in the range of 1.50–1.64) resulting in either partial or total transformation into β-TCP [$Ca_3(PO_4)_2$] during calcination at 700–750 °C to crystallize all amorphous phases.

The previous studies indicate a need for considerable improvement of the mechanochemical and mechanochemical-hydrothermal synthesis methods of HAp in terms of crystallinity, stoichiometry, and deagglomeration. The aim of the present study was synthesis of well-crystallized HAp powders with precisely controlled chemical composition by the mechanochemical-hydrothermal method.

Calcium hydroxide powder [$Ca(OH)_2$] and solid diammonium hydrogen phosphate [$(NH_4)_2HPO_4$] (analytical grade, Alfa Aesar, Ward Hill, MA) were used as reactants for synthesis of HAp. Their purity was confirmed by x-ray diffraction and thermogravimetric analysis. A total of 50 batches of the HAp powder were prepared. Each batch was prepared according to the following procedure. First, a suspension of 25.251 g $Ca(OH)_2$ in 350 ml of deionized water was prepared in a 500 ml glass beaker. Subsequently, 26.736 g of $(NH_4)_2HPO_4$ powder was slowly added to the same beaker at constant vigorous stirring using a magnetic stirrer for about 10 min. In one experiment, 2-propanol (C_3H_7OH, histological grade, Fisher Scientific, Pittsburgh, PA) was used instead of water for comparison purposes while maintaining all other preparation conditions as before. The Ca/P molar ratio in each starting slurry was 1.67, corresponding to stoichiometric HAp. The mechanochemical-hydrothermal synthesis was performed by placing slurries into a laboratory-scale mill (model MIC-0, NARA Machinery Co., Tokyo, Japan) equipped with a zirconia liner and zirconia ring grinding media. The milling equipment is a multi-ring media mill. Its grinding mechanism is different than those used for attrition, planetary, or vibratory mills, as described elsewhere.[22]

Grinding of the slurries was carried out in air, initially at a rotation speed of 1500 rpm for 1 h and then at 800 rpm for 4 h. Temperature was measured during the grinding using a thermocouple. It ranged between 29 and 35 °C at 1500 rpm and 25 and 32 °C at 800 rpm depending upon the batch. The solid phase was washed free of residual species using distilled water after the mechanochemical-hydrothermal synthesis and subsequently dried either in an oven at 70 °C for 24 h or by freeze drying (VirTis Freezemobile 6 with unitop 600SL, VirTis, Gardiner, NY). In the case of freeze drying, the slurry was first frozen in liquid nitrogen, then fractured into 1-in. or smaller chunks and placed into the freeze-drier with a shelf at −10 °C under vacuum of approximately 60 mtorr and held for 2 days under such conditions. Subsequently, the temperature was raised to 25 °C, and kept constant for 1 day. A typical batch of a dry HAp powder was about 30 g. To check thermal stability of the prepared HAp phase herein referred to as "heat-treated," a small quantity of each as-prepared HAp powder was heat treated in air at 900–1100 °C for 1 h with a heating rate of 10 °C/min.

All batches of as-prepared and heat-treated HAp powders were characterized by x-ray diffraction (XRD, Kristalloflex D-500, Siemens Analytical X-ray Instrument Inc., Madison, WI) using Ni filtered Cu K_α radiation, in the 2θ range of 10–70° at a scan rate of 2.4 °/min, with a sampling interval of 0.05°.

Specific surface area of randomly selected batches of as-prepared and heat-treated HAp powders was measured using the Brunauer–Emmett–Teller (BET) method utilizing adsorption of N_2 gas (purity 99.99%; Matheson Co., Bridgeport, NJ) at −196 °C (Coulter Surface Area Analyzer SA 3100, Coulter Co., Fullerton, CA).

Particle size distributions for randomly selected batches of as-prepared HAp powders was determined by dynamic light scattering at a wavelength of 632.8 nm (DLS, model DLS-700, Otsuka Electronics Co., Osaka, Japan). Samples for the DLS measurements were prepared by dispersing small amounts of the HAp powder in ethanol (filtered using a 0.2-μm filter) followed by immersion into an ultrasonic bath for 10 min.

Size and degree of agglomeration of the synthesized particles for randomly selected batches of as-prepared HAp were studied using field emission scanning electron microscopy (FESEM) at 1–6 kV with a working distance of 2–16 mm (Model DSM 962, Gemini, Carl Zeiss, Inc., Thornwood, NY). Infrared spectrum of a selected batch of as-prepared HAp was obtained using an infrared Fourier-transform spectrometer (FTIR, model 1720-X, Perkin Elmer Co., Norwalk, CT). For this purpose, the HAp powder was mixed using a mortar and pestle with KBr in the proportion 1:150 (by weight) for 15 min and pressed into a pellet using a hand press. Weight changes of the as-prepared HAp powder from a selected batch have been measured at a heating rate of 5 °/min in flowing air (30 ml/min.) over a temperature range of 25–1000 °C using a thermogravimetric analyzer (model TGA-7, Perkin Elmer Co., Norwalk, CT). Chemical analysis of selected batches of the as-prepared HAp for calcium and phosphorus was accomplished by x-ray fluorescence (XRF; at Oneida Research Services, Inc. Whitesboro, NY), and for carbonate by carbon coulometry.

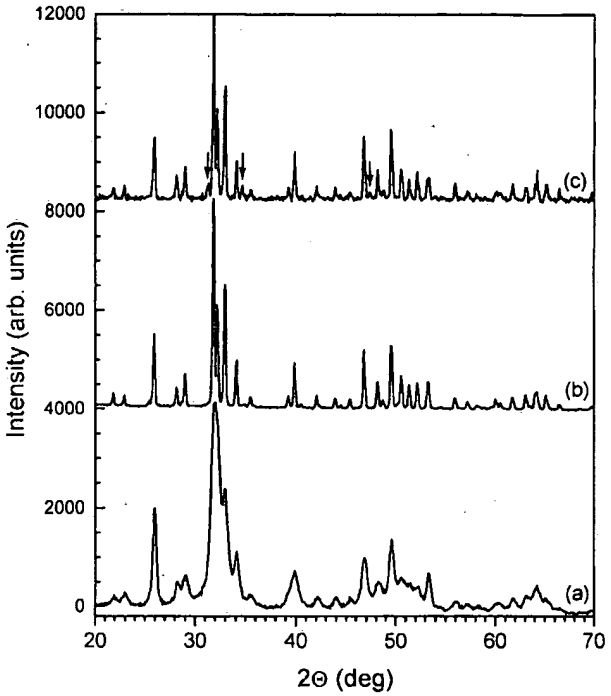

FIG. 1. XRD patterns of the HAp powders (a) as-prepared, (b) after calcination in air at 900 °C for 1 h, and (c) after calcination in air at 1100 °C for 1 h. Arrows indicate strongest peaks derived from β-TCP. Unmarked peaks are derived from HAp.

A typical x-ray diffraction (XRD) pattern of the as-prepared HAp powder is shown in Fig. 1(a). The XRD peaks are well defined and attributable only to HAp lattice planes. Heat treatment of the as-prepared HAp powders at 900–1100 °C for 1 h in air further narrowed their diffraction peaks [Figs. 1(b) and 1(c)]. No peaks attributed to either β–TCP [the strongest (0120) peak at $2\theta \approx 31°$] or CaO [the strongest (200) peak at $2\theta \approx 37.5°$] were observed in the HAp powders heat-treated at 900 °C [Fig. 1(b)]. Low-intensity β–TCP peaks appeared in the XRD patterns of the HAp powder heat treated at 1100 °C [Fig. 1(c)]. Comparison of integrated intensities of strongest HAp and β–TCP peaks in the HAp powders heat treated above 800 °C has been known as a simple method to estimate the Ca/P molar ratio.[6] The XRD analysis of the HAp powders prepared by the mechanochemical-hydrothermal route indicates that it is slightly Ca-deficient with a chemical composition close to the stoichiometric one (i.e., Ca/P ≈ 1.66 to 1.67). XRF measurements confirmed the XRD measurements by yielding a Ca/P molar ratio of 1.76 ± 0.1, which, based on the experimental error bracketed the stoichiometric Ca/P molar ratio of 1.67.

The FTIR spectrum of the as-prepared HAp powder is shown in Fig. 2. It is a typical spectrum of HAp showing PO_4-derived bands at 478, 566, 605, 963, and 1030–1090 cm^{-1}, and OH-derived bands at 630 and

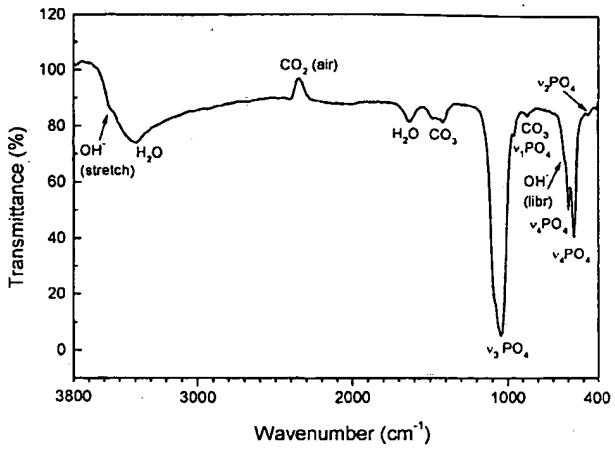

FIG. 2. FTIR spectrum of the as-prepared HAp powder.

3570 cm^{-1}.[8] The bands derived from carbonate ions (CO_3^{2-}-for-PO_4^{3-} substitution) are present at 870 cm^{-1} and around 1420–1480 cm^{-1}.[8] The carbonate concentration was 0.8 wt%, as determined by carbon coulometry with an estimated error of ± 5% of the measured value. Adsorbed water bands are located at 1630 and 3000–3700 cm^{-1}. The low intensity of both OH-derived bands may be due to large specific surface area of the HAp powders, the presence of adsorbed water, and incorporation of small quantities of carbonate ions in the HAp lattice.[6]

Thermogravimetric analysis of the as-prepared HAp powder in the temperature range of 25–1000 °C confirmed results of the other characterization techniques. Significant loss of weight was observed up to approximately 450 °C and could be due to the loss of adsorbed and lattice water.[8] Above this temperature, our HAp exhibited minimal weight loss, which is typical for stoichiometric HAp.[8]

Specific surface area of the as-prepared HAp powders ranged between 89–93 m^2/g (± 2 m^2/g), which corresponds to an estimated equivalent spherical diameter (d_{BET}) of 20–21 nm. Thus it appears that the very small crystallite size caused broadening of the XRD peaks [Fig. 1(a)], as opposed to lattice disorder. Measurements of the particle size distribution bracketed the range of 0.15–3.0 μm. The number-based particle size distribution was single-modal with an average value of 685 nm. These measurements indicate the presence of aggregates and/or agglomerates consisting of hundreds of primary particles per average aggregate/agglomerate. This was confirmed by FESEM observations shown in Fig. 3. The as-prepared HAp powder contained large agglomerates, 1–2 μm in diameter, which consisted of very fine HAp crystals (Fig. 3).

The mechanochemical-hydrothermal synthesis of HAp using $Ca(OH)_2$ and $(NH_4)_2HPO_4$ is a solution-mediated reaction. $(NH_4)_2HPO_4$, which is highly soluble in water,

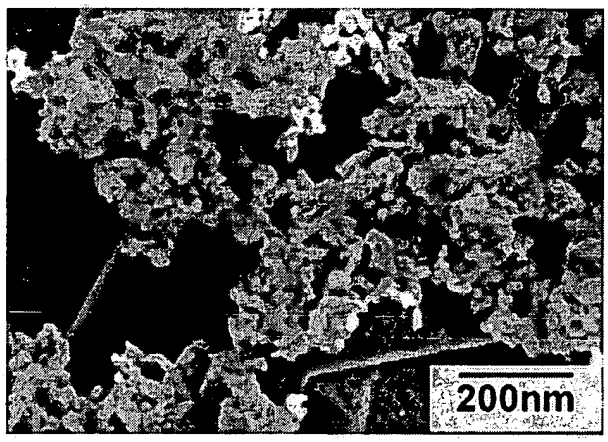

FIG. 3. FESEM photograph of the as-prepared HAp powder showing agglomeration of nano-sized HAp primary particles.

reacts with solid $Ca(OH)_2$ whose dissolution rate limits the reaction. However, given the solubility of $Ca(OH)_2$ and the influence of mechanical activation, the rate of this process is rapid enough to be conducted at room temperature. Our results emphasize importance of the aqueous solution, which actively participates in the synthesis reaction by dissolving one of the reactants, which is not observed with conventional mechanochemical synthesis of HAp. The experimental conditions utilized in previous research,[11–18] differed considerably but also shared certain general features. All reactants either exhibited very low solubility in water (0.00014–0.0655 m), or the mechanochemical treatment was accomplished under dry conditions. All HAp powders synthesized with wet or dry approaches were highly nonstoichiometric and had very low crystallinity. On the other hand, in the present work, one of the reactants, $(NH_4)_2HPO_4$, exhibited very high solubility in water (4.35 m) and the HAp powders synthesized using that reactant were nearly stoichiometric and had high crystallinity and thermal stability.

To test our understanding of the reaction mechanism, we chose 2-propanol as a solvent for mechanochemical reaction, which is known to be a poor solvent for $Ca(OH)_2$ and $(NH_4)_2HPO_4$.[23] This solvent is also known to crystallize HAp at elevated temperature (200 °C).[24] However, when 2-propanol was substituted as a solvent instead of water, under otherwise equal conditions no HAp was observed to form. Therefore we believe that the crystallization of HAp under mechanochemical-hydrothermal conditions is most likely a dissolution-rate-limited process.

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